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Regulatable systemic production of monoclonal antibodies by *in vivo* muscle electroporation

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Abstract

The clinical application of monoclonal antibodies (mAbs) potentially concerns a wide range of diseases including, among others, viral infections, cancer and autoimmune diseases. Although intravenous infusion appears to be the simplest and most obvious mode of administration, it is very often not applicable to long-term treatments because of the restrictive cost of mAbs certified for human use and the side effects associated with injection of massive doses of antibodies. Gene/cell therapies designed for sustained and, possibly, regulatable *in vivo* production and systemic delivery of mAbs might permit to advantageously replace it. We have already shown that several such approaches allow month- to year-long ectopic antibody production by non-B cells in living organisms. Those include grafting of *ex vivo* genetically modified cells of various types, *in vivo* adenoviral gene transfer and implantation of encapsulated antibody-producing cells. Because intramuscular electrotransfer of naked DNA has already been used for *in vivo* production of a variety of proteins, we have wanted to test whether it could be adapted to that of ectopic mAbs as well. We report here that this is actually the case since both long-term and regulatable production of an ectopic mAb could be obtained in the mouse taken as a model animal. Although serum antibody concentrations obtained were relatively low, these data are encouraging in the perspective of future therapeutical applications of this technology in mAb-based immunotherapies, especially in developing countries where cost-effective and easily implementable technologies would be required for large-scale applications in the context of severe chronic viral diseases such as HIV and HCV infections.

Findings

The therapeutical potential of monoclonal antibodies (mAbs) is enormous. Twelve mAbs have already been approved by the US Food and Drug Administration for therapeutical use and 400 others are currently under clinical evaluation [1,2]. However, a number of hurdles have still to be overcome before efficient therapeutical application of mAbs on large scales and at reasonable costs. This

is particularly true in the case of chronic diseases where patients must be treated for years or even for their whole lifetime. If mAb intravenous injection is a suitable mode of administration for short-term treatments, this is often not the case for long-term ones mainly because of (i) the mild to severe side effects associated with infusion, (ii) the possible anti-idiotypic response resulting from repeated injections of massive doses of antibodies and (iii) the restrictive costs of *in vitro* produced proteins certified for human use. Moreover, injection of massive doses of mAbs results in great variations in the bioavaibility of these therapeutic agents that are often detrimental to the efficacy of treatments. It is, therefore, important to investigate whether *in vivo* production of therapeutic antibodies based on gene/cell therapy-based approaches can advantageously replace regular intravenous infusions. This would render long-term therapeutic antibody treatments cost-effective, eliminate side effects of infusions and lower, or delay, the antibody-neutralizing response of the host through continuous and sustained delivery of antibodies at relatively low, but therapeutic, levels (for a review, see [3]).

Several methods have already been used to achieve month- to year-long ectopic mAb production in the mouse taken as an animal model. Those include: (i) grafting of *ex vivo* modified myoblasts [4], skin fibroblasts [5] and skin patches [6], (ii) intravenous and intramuscular injection of recombinant adenoviral vectors [7], (iii) intramuscular injection of AAV vectors [8] and (iv) implantation of mAb-producing cells encapsulated in an immunoprotective matrix made of cellulose sulphate [9]. Remarkably, the latter technology allowed to cure retrovirally-infected mice from a lethal neurodegenerative disease upon production of a neutralizing mAb [10] thereby demonstrating the therapeutical interest of the approach.

Neutralizing anti-HIV and anti-HCV antibodies, some of which are currently tested in the clinic [11,12], might potentially be of great help to fight two major health concerns of developing countries, provided that cost-effective and easily implementable methods of administration are available. Because the above-mentioned techniques, even in optimized forms, would certainly not meet these criteria, we turned to gene transfer methods as simple and as inexpensive as possible in the perspective of long-term therapies. Taking into account that muscle cells are competent for synthesis and secretion of properly folded mAbs [4,7,8], we first tested intramuscular injection of naked plasmid vectors with, however, no success (not shown). We then considered skeletal muscle DNA electrotransfer, a physical method for DNA delivery based on intramuscular injection of plasmids followed by electric pulses delivery [13-15], since it was reported to be more efficient than injection of DNA alone for systemic production at a therapeutic level of a number of proteins such as erythropoietin, factor IX and cytokines (for reviews see [16,17]). The higher efficiency of DNA electrotransfer appears to be a two component phenomenon involving, on one hand, cell permeabilization and, on the other hand, DNA electrophoresis [18-20]. Thus far, DNA electrotransfer has been used with success in different species including mouse, rat, dog and monkey, but has not been reported yet in humans. It is, however, important to underline that, in addition to its simplicity, this approach should also easily permit multivalent one-step treatments through the mixing of different expression vectors. In the specific case of mAb-based antiviral treatments, this would offer the advantage of limiting the risk of viral escape.

In a first step, we tested whether long-term mAb production could be obtained using constitutive expression vectors. The cDNAs for heavy (hTg10) and light (κTg10) chains of the Tg10 mouse mAb (IgG2a/κ; [21]) were cloned downstream of a CMV promoter in the pCOR vector backbone [22] to give pCOR-κTg10 and pCOR-hTg10 plasmids, respectively (Figure 1A). Tg10 is directed against human thyroglobulin and was selected because of its easy assay in ELISA. The advantages of the pCOR vector are multiple: (i) it contains a conditional replication origin permitting its production only in specifically engineered *E. coli* strains to reduce the risks of uncontrolled propagation in the environment and in treated patients, (ii) it can be produced in high yields through fed-batch fermentation, (iii) it contains minimal amounts of procaryotic sequences to minimize interferences with transgene expression (by reducing the immune response) and (iv) it already allowed substantial transgene expression upon injection into mouse skeletal muscle [22]. Five C57Bl6 mice were subjected to intramuscular electroporation with an equimolar mixture of pCOR-κTg10 and pCOR-hTg10 and five others, taken as negative controls, with a saline solution. Tg10 levels in the bloodstream were subsequently followed up as a function of time. No Tg10 was detected in control mice whereas all other mice expressed it for at least 142 days (duration of the experiment) (Figure 1B). Although variations between animals were observed, it is noteworthy that 3 of them expressed more than 1 μg/ml in the initial production period under non-optimized experimental conditions (see below).

Because regulatable expression would eventually be desirable to adapt serum mAb levels to the patients' needs or to terminate treatments in case of adverse side effects, we next turned to the use of the tet-off and tet-on inducible systems developed by Bujard and collaborators (for a review, see [23]). In both of them, transgene transcription is under the *cis*-control of a minimal CMV promoter linked to multiple copies of the bacterial tetracycline

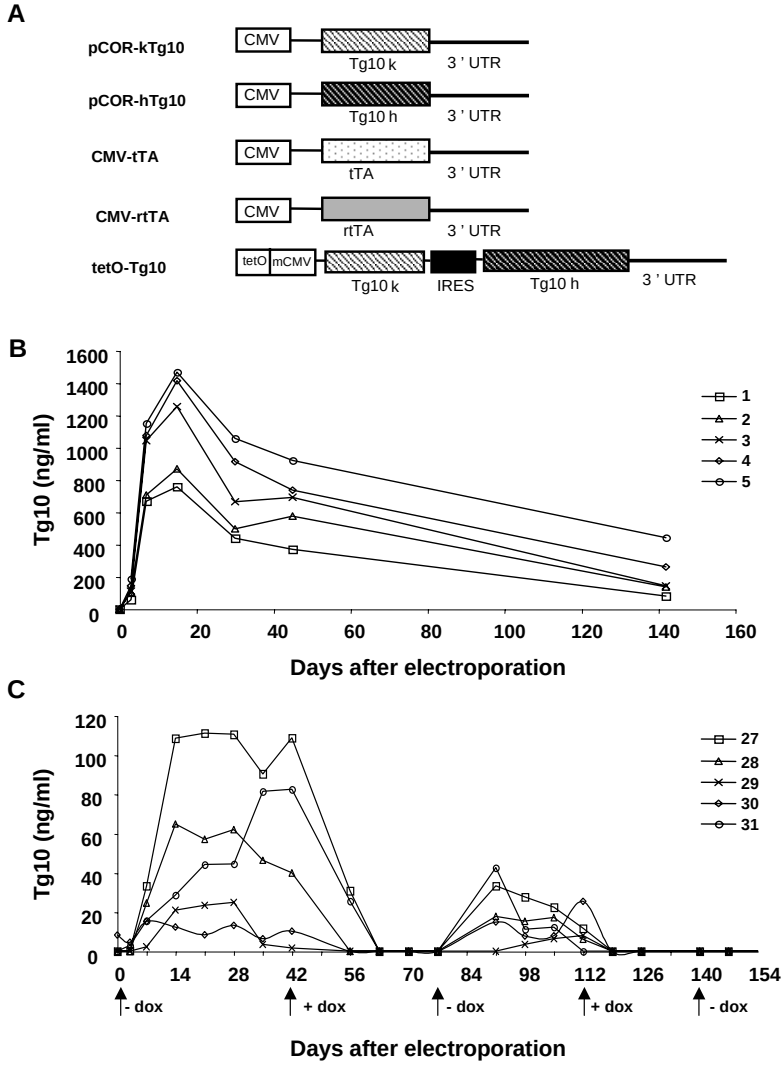

Figure 1

Tg10 mAb production in mice subjected to intramuscular electrotransfer. *(A) Tg10 mAb-expressing vectors used for electroporation*. pCOR-κTg10 and pCOR-hTg10, pCOR-derived vectors expressing the Tg10 light (κTg10) and Tg10 heavy (hTg10) chains cDNAs under the control of the CMV promoter, respectively. CMV-tTA and CMV-rtTA express tTA and rtTA transactivators under the transcriptional control of the CMV promoter, respectively. In tetO-Tg10, κTg10 and hTg10 are expressed from a monocistronic expression cassette owing to the poliovirus internal ribosome entry sequence (IRES) placed under the cis-control of a minimal CMV promoter linked to multiple copies of the bacterial tetO operator [24]. *(B):* in vivo *Tg10 production after electroporation of pCOR-derived vectors*. Ten 4 week-old C57Bl6 mice were divided into 2 groups and injected intramuscularly in the *tibialis anterior* with either 20 μg of pCOR-κTg10 plus 20 μg of pCOR-hTg10 (mice 1 to 5) or a saline solution taken as a negative control. Electroporation was then performed as described in [15]. Blood samples were withdrawn at the indicated time points post-electroporation and serum Tg10 levels were assayed by ELISA [4]. (*C): Regulatable* in vivo *Tg10 mAb production*. 4 week-old C3H mice were divided into 3 groups of 5 animals and injected intramuscularly in the *tibialis anterior* with either a saline solution (not shown), 100 μg of tetO-Tg10 (not shown) or 100 μg of CMV-tTA plus 100 μg of tetO-Tg10 (mice 27 to 31). Electroporation was then performed as described in [15]. Doxycycline was added or removed from mice drinking water at indicated times. Tg10 levels in serum samples taken at different time points post-electroporation were assayed by ELISA.

operator (TetO). Expression is controlled by a transactivator (tTA) negatively regulated by tetracycline, or some of its derivatives such as doxycycline (Dox), in the tet-off system, whereas it is dependent on a transactivator (rtTA) positively regulated by tetracycline family antibiotics in the tet-on one. A monocistronic expression cassette carrying κTg10 and hTg10 cDNAs separated by the poliovirus IRES was thus cloned downstream of TetO in the pUHD10-3 vector [24] to give the tetO-Tg10 plasmid (Figure 1A). In a first series of experiments, four groups of 4 mice were subjected to intramuscular DNA electrotransfer with: (i) a saline solution, as a negative control, (ii) 25 μg of tetO-Tg10 alone, to detect possible leakiness of the expression system, (iii) 25 μg of both tetO-Tg10 and a constitutive expression vector for tTA (CMV-tTA; Figure 1A) and (iv) 25 μg of both tetO-Tg10 and a constitutive expression vector for rtTA (CMV-rtTA; Figure 1A). Tg10 was expressed in none of the mice treated with the saline solution or with tetO-Tg10 alone (not shown). Similarly, in the presence of Dox in the drinking water, Tg10 was detected in none of the mice electroporated with CMV-rtTA and tetO-Tg10 (not shown). In contrast, in the absence of doxycycline, mice injected with CVM-tTA plus tetO-Tg10, presented low, but detectable, serum levels (10–20 ng/ml) of Tg10 one month after electroporation, at which time the experiment was stopped. The better production observed with the tet-off system is consistent with *in vitro* transfection experiments previously performed with mouse myogenic C2.C12 cells (not shown). The tet-on system was therefore not considered any longer for further work. In a second series of experiments, we tested whether electroporation of higher quantities of DNA could lead to higher levels of mAb serum levels (Figure 1C). Three groups of 5 mice each were treated with either a saline solution, 100 μg of tetO-Tg10 alone or 100 μg of CVM-tTA plus 100 μg of tetO-Tg10. No Tg10 was detected in control mice (not shown). In the absence of doxycycline, the mice injected with CMV-tTA plus tetO-Tg10 showed a mAb production increasing for the first to weeks, at which time it was more or less stabilized at levels ranging from 10 to 110 ng/ml depending on the mouse. On day 42, Dox was added to the drinking water, which led to rapid Tg10 production shut-off, and removed on day 77, which permitted Tg10 reinduction albeit at a lower degree than in the first period of expression. A new cycle of repression/reinduction was attempted upon addition and removal of Dox on days 112 and 140, respectively, with, however, no success (Figure 1C). Taken together these data indicate that higher mAb production can be obtained upon electroporation of higher amounts of expression vectors and that regulated expression can be obtained, at least as long as the inducible system remains functional (see discussion below).

In conclusion, we report here that intramuscular electroporation of naked DNA allows both constitutive and regulatable *in vivo* production of ectopic mAbs, which constitutes a first step towards simplified, multivalent and cost-effective long-term mAb-based genetic immunotherapies. Although month-long mAb expressions could be observed, production levels remained low, indicating that improvements of the technology are required before efficacious and reliable human application. We and others have previously shown that muscle cells can achieve high antibody production when genetically modified by adenoviral or AAV vectors ([7,8] and unpublished data). Thus, the low mAb serum levels observed here were not due to a limited ability of muscle cells to secrete mAb but rather to the electroporation method itself and/or to the poor efficiency of the expression vectors used. Optimizing electric pulses for better adaptation to muscle geometry as well as improving plasmid biodistribution will thus have to be considered to improve DNA electrotransfer. Similarly, the search for optimal DNA doses will have to be conducted as our own data also indicate that the quantity of expression vectors is critical with regard to the final mAb expression. Finally, further improvements might also come from the optimization of (i) the plasmid structure itself, to eliminate intrinsic immunostimulatory sequences as much as possible, and (ii) the expression cassette for better transcription, translation and secretion of antibodies by muscle cells [25,26]. Along this line, rather than utilizing the CMV promoter, which is known to undergo progressive shut-off *in vivo* and to display relatively modest activity in a variety of tissues [27], using strong muscle-specific promoters such as the muscle-specific creatine kinase- [28], desmin- [29] or synthetic pSPc5-12 promoter developed by Li and collaborators [30] should reveal particularly rewarding. In this regard, preliminary *in vitro* studies showed that pSPc5-12 promoter allows 100-fold higher expression of a luciferase gene than the CMV promoter in differentiated muscle cells (NP, unpublished data). Concerning the regulatable tetracycline system used here, we were not able to reactivate Tg10 production a third time. The reasons for this are not yet clear. Whether this was due to an immune response mounted against the tetracycline-dependent transactivator as has already been reported elsewhere for rtTA ([31] and NP unpublished observations), to the instability of the DNA vectors used or to gene expression shut-off resulting from inactivation of the CMV promoter remains to be evaluated. It is, however, of note that longer term regulatable expression of other recombinant proteins such as erythropoietin upon MLV- and AAV-based transduction of muscle cells has been described [31-34]. In these experiments, transcription of tTA and rtTA genes was driven either by the muscle-specific desmin promoter or by a viral LTR promoter, suggesting that the choice of the promoter might be crucial for long-term expression of

tetracycline-dependent transactivators in muscle electroporation settings. Testing other muscle-specific promoters for tTA and rtTA, as well as other inducible systems such as the rapamycin inducible system is currently underway towards this aim.

Competing interests

None declared.

Authors' contributions

NP carried out and participated in the design of DNA electrotransfer experiments allowing regulatable Tg10 expression. PB constructed pCOR-derived vectors expressing Tg10 and performed pCOR electrotransfer experiments. DS and OD participated in the design of the study. MP and MPelegrin participated in the design and coordination of the study and drafted the manuscript. MPelegrin also performed *in vitro* transient transfection experiments and Tg10 ELISA assays. All authors read and approved the manuscript.

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